The Viability of Cytochalasin B and Heat Shock Induction for the Production of Triploid *Mytilus Edulis*

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2005

Canadian Technical Report of Fisheries and Aquatic Sciences 2572

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To whom it may concern,

Please add this label to the front cover of recently received "The Viability of Cytochalasin B and Heat Shock Induction for the Production of Triploid *Mytilus Edulis* - Canadian Technical Report of Fisheries and Aquatic Sciences 2527". The # that was placed on the front cover is incorrect and needs to be changed to 2572.

I apologize for the error and hope that this change did not cause you too much inconvenience.

Regards,

Andrew Cogswell

ERRATUM

Cogswell, A.T., E.L. Kenchington, B.W. MacDonald and S.E. Roach. 2005. The viability of cytochalasin B and heat shock induction for the production of triploid *Mytilus edulis*. Can. Tech. Rep. Fish. Aquat. Sci. 2572: v + 32 p.

Page ii,iv and v: correct citation for this publication should read:

Cogswell, A.T., E.L. Kenchington, B.W. MacDonald and S.E. Roach. 2005. The viability of cytochalasin B and heat shock induction for the production of triploid *Mytilus edulis*. Can. Tech. Rep. Fish. Aquat. Sci. 2572: v + 32 p.

105-05703/

Canadian Technical Report of
Fisheries and Aquatic Sciences 2527

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by

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Cat. No. Fs 97-6/2527E ISSN 0706-6457

Correct citation for this publication:

Cogswell, A.T., E.L. Kenchington, B.W. MacDonald and S.E. Roach. 2005. The viability of cytochalasin B and heat shock induction for the production of triploid *Mytilus edulis*. Can. Tech. Rep. Fish. Aquat. Sci. 2527: v + 32 p.

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ABSTRACT

Cogswell, A.T., E.L. Kenchington, B.W. MacDonald and S.E. Roach. 2005. The viability of cytochalasin B and heat shock induction for the production of triploid *Mytilus edulis*. Can. Tech. Rep. Fish. Aquat. Sci. 2527: v + 32 p.

The Maritime blue mussel (*Mytilus edulis*) aquaculture industry has seen a ~75% increase in production since 1992 (DFO, 2003); thus, government and industry are currently vested in research designed to optimize production. One area of research interest is the feasibility of mass producing triploid (3N) *M. edulis* via heat shock or chemical induction with Cytochalasin B (CB). The one heat shock trial was attempted using eggs from a hybrid (*M. edulis* x *M. trossolus)* female fertilized with pooled sperm from 5 pure *M. edulis* males. Fertilized eggs exposed to a 30.8°C water bath for 10 minutes, 20 minutes post-fertilization resulted in a ~70% triploid induction rate when assessed only 4 hours after fertilization. Chemical (CB) triploidy induction trials were attempted using one *M. edulis* male to separately fertilize the eggs of three *M. edulis* female broodstock. Exposing fertilized eggs to 0.5 mg/l CB to inhibit the second meiotic division resulted in 95-100% triploids when sub-samples were tested from 2 to 37 days post-spawn. Preliminary growth trials revealed that there is no significant differences in growth rate between diploids (2N) and triploids (3N) (<1 to 20 mm shell height) produced with CB. Efficient production of triploid *M. edulis* will allow researchers to do further in-depth metabolic and morphometric studies in conjunction with growth, survival and overall health associated with a variety of field conditions in order to assess the commercial applicability of triploid *M. edulis*. This information can also be used to guide regulations for the introduction and transfer of triploid mussels.

RÉSUMÉ

Cogswell, A.T., E.L. Kenchington, B.W. MacDonald and S.E. Roach. 2005. The viability of cytochalasin B and heat shock induction for the production of triploid *Mytilus edulis*. Can. Tech. Rep. Fish. Aquat. Sci. 2527: v + 32 p.

L'industrie aquacole des Maritimes a vu la production de la moule bleu (*Mytilus edulis*) augmenter d'environ 75% depuis 1992 (MPO, 2003); ainsi, le gouvernement et l'industrie coopèrent actuellement sur des projets de recherche désignés à optimiser la production. Un point d'intérêt de la recherche est la faisabilité de produire en masse des (*M. edulis*) triploïdes (3N) par chocthermique ou par induction chimique avec la Cytochalasine B (CB). Un essai d'induction par chocthermique a été réalise en utilisant les œufs d'une femelle hybride (*M. edulis x M. trossolus*) et le sperme d'un male pur *M. edulis*. L'exposition des œufs 20 min après fertilisation a 30.8°C pendant 10 min a résulte en ~70% de triploïdes, 4 heures après fertilisation. Les essais d'induction de triploïdes par induction chimique (CB) ont été réalises en utilisant un *M. edulis* mâle pour fertiliser séparément les œufs de trois femelles *M. edulis*. L'exposition a la CB à une concentration de 0.5mg/l pour bloquer la deuxième division méiotique, a résulté en 95-100% de triploïdes, ceci de 2 à 37 jours après la ponte. Les essais préliminaires de croissance nous ont montré qu'il n'y avait pas de différence significative du taux de croissance entre les diploïdes et les triploïdes de < 1 à 20 mm de hauteur de coquille fabriqué avec CB. Des études plus profondes sur le métabolisme et la morphologie des *M. edulis* en conjonction avec la croissance, la survie et la santé générale associés avec une variété de conditions vont donner aux chercheurs une vue plus compréhensive de l'application commerciale des *M. edulis* triploïdes. Cette information peut être aussi utilisée comme guide pour les règlements sur l'introduction et le transfert des moules triploïde.

DEFINITION OF TECHNICAL TERMS

Adipogranular Cells – Energy storage cells observed in the connective tissue between gonadal follicles.

Aneuploid – Possessing a number of chromosomes which is not an exact multiple of the haploid chromosome number.

Diploid (2N) – Possessing 2 sets of chromosomes.

Electrofusion – electrical stimulation of early embryos which fuses dividing cells resulting in polyploidy.

Hemocytes – Blood cell found in the hemolymph. There are several types that perform a wide variety of functions (e.g., phagocytosis and nutrient transport).

Mosaic – Descriptive of an organism containing cells of different ploidies.

Tetraploid (4N) – Containing 4 sets of chromosomes.

Triploid (3N) – Containing 3 sets of chromosomes (typically by retention of 2^{nd} polar body).

INTRODUCTION

According to Statistics Canada, Canadian aquaculturists produced ~ 20,540 tons of blue mussels (*M. edulis*) in 2002. Although this is less than 2% of the 1.447 x 10^6 tons produced worldwide through mussel culturing in 2002, it was still worth nearly $31,500,000 predominately to maritime aquaculturists, who in PEI alone hold approximately 80% of our national production (FAO 2004; Statistics Canada 2003; McDonald *et al.* 2002). As an industry that has seen a ~75% increase in production since 1992 (Statistics Canada 2003), both government and industry are vested in research designed to optimize production and maximize profits. Much of the current production is focused on the collection of wild-caught spat; however, with the low end market established, growers are becoming more interested in the possibility of producing hatchery-reared animals which in theory would reduce the annual variability associated with current collection methodology. The Invertebrate Fisheries Division (IFD) of DFO is currently exploring biotechnological techniques that may have direct research and commercial applicability. This report specifically focuses on the commercial viability and research applicability of the mass production of triploid (3N) blue mussels via chemical induction with cytochalasin B (CB).

TRIPLOIDS: INCEPTION AND APPLICABILITY

Stanley *et al.* (1981) published the first paper describing 3N shellfish production for the American oyster (*Crassostrea virginica*). Initial interest in 3N shellfish has likely stemmed from observed growth increases for some scallop, oyster and clam species (i.e., *Argopecten irradians/ventricosus*, *C. virginica* and *Mulinia lateralis*) (Ruiz-Verdugo *et al.* 2000; Guo and Allen 1994a; Stanley, 1984; Tabarini 1984); however, 3N sterility and the resultant meat quality improvement during peak spawning season for some species (*C. gigas, Mytilus galloprovincialis,* and *Mytilus edulis*) have been crucial factors spurring further research into the viability of commercial 3N production (Brake *et al.* 2004; Nell 2002; Davis 1997; Allen 1987).

Unlike diploid (2N) shellfish, 3N's are gametogenically suppressed due to their inability to properly synapse during meiosis (Beaumont and Fairbrother 1991). Triploid mussels (*M. galloprovincialis*) are nearly 100% male and never fully mature (Kiyomoto *et al.* 1996). In fact, after an initial stage of "spermatogonia proliferation", the appearance of hemocytes leads to resorbtion of gonadic tissue and the re-emergence of adipogranular cells commonly seen in 2N gonadic tissue outside of the typical spawning season (Kiyomoto *et al.* 1996) (Appendix 1). According to Beaumont and Fairbrother (1991) sterility is commercially important for 3 main reasons: First, energy in the form of glycogen usually reserved for gonadal development can be redirected to somatic tissue leading to elevated somatic glycogen content and increased somatic growth. Second, poor condition of ne species (*M. edulis* and *C. gigas)* pre- or post-spawn will negatively impact appearance, taste and/or meat yield, thus reducing their marketability (Brake *et al.* 2002; Allen and Downing 1991). Triploid shellfish mitigate this problem due to retarded gonadal development and the subsequent somatic reallocation of glycogen reserves which in some cases leads to improved taste and year-round

marketability (Allen and Downing 1991). Thirdly, when intentionally or incidentally introduced into established local populations, sterile triploid organisms would reduce introgression of foreign genes (i.e. hybridization).

TRIPLOID INDUCTION TECHNIQUES

Since 1981, 3N production methods in many species of oysters, clams, scallops and mussels have been described and refined (Allen 1987; Beaumont and Fairbrother 1991; Desrosiers *et al.* 1993; Scarpa *et al.* 1994; Davis 1997; Jackson *et al.* 2003). The most commonly employed 3N shellfish induction procedure involves retaining the 2nd polar body of the early embryo after fertilization via chemical or physical treatment. Techniques employed include: electrofusion, cold/heat shock, caffeine, cytochalasin B (CB), 6-dimethylaminopurine (6-DMAP), and nearly any combination of the aforementioned examples (Cadoret 1992; Yamamoto and Sugawara 1988; Scarpa *et al.* 1994; Toro and Sastre 1995; Davis 1997). Until recently, the most successful and commercially viable method of 3N production involved treating fertilized eggs with CB, a cell permeable fungal toxin (Forscher and Smith 1988; Allen *et al.* 1989). CB, by inhibiting actin polymerisation and halting microtubule formation, retains the 2nd polar body of the developing embryo, thus producing an individual with 2 maternal and 1 paternal chromosome complement. Occasionally however, the retention of this extra chromosomal set is incomplete resulting in aneuploid individuals which rarely persist through D stage metamorphosis (Guo *et al.* 1990). Although 3N induction success utilizing CB has been highly variable between species (Beaumont and Fairbrother 1991), 3N's are produced in enough quantity that this method is commercially viable. In fact, nearly 30% of all Pacific oysters farmed on the West Coast of North America in 1999/2000 were 3N's produced by this method (Nell 2002). Previous 3N induction of *M. galloprovincialis* and *M. edulis* utilizing CB (1 mg/l) has been variable (67-86%) (Beaumont and Kelly 1989; Scarpa *et al.* 1994). However, current CB 3N induction trials with *M. edulis* at the Bedford Institute of Oceanography using 0.5 mg/l CB have been quite successful (95-100% induction).

As well, heat shock (32°C for 10 minutes at 20 minutes after fertilization), has been used by Yamamoto and Sugawara (1988) to produce high percentages (97%) of 3N offspring. While further attempts by other researchers using the methodology employed by Yamamoto and Sugawara (1988) has lead to less impressive results with *Mytilus sp.* (15-84% 3N induction), these poor results were likely due to the timing and duration of heat shock exposure (Toro and Sastre 1995; Scarpa *et al.* 1994). A preliminary trial at the Bedford Institute of Oceanography employing the latest in induction techniques was conducted to assess the viability of 3N induction via heat shock.

The most reliable method of 3N production involves crossing tetraploid (4N) male broodstock with diploid (2N) females to produce 100% 3N offspring (Eudeline *et al.* 2000). Currently, technology developed to successfully produce commercial quantities of 4N Pacific and Atlantic oysters (*C. gigas and C. virginica*) is proprietary/patented and owned by 4C's Breeding Technologies, Inc (Wildwood, New Jersey). The most successful techniques employed to produce 4N broodstock, at least in Pacific oysters, requires inhibiting the first polar body extrusion of eggs from 3N females

fertilized with sperm from 2N's by treating them with 0.5 mg/L CB added ~10 minutes after fertilization (Eudeline *et al.* 2000; Guo and Allen 1994b). As of yet, only putative eggs have been identified in 3N *M. edulis* (Brake *et al.* 2004) and these are rarely ever seen (<1%) due to the high incidence of males (~100%) in confirmed 3N offspring; however, Scarpa *et al.* (1993) exposed fertilized eggs from 2N females to 1 mg/l CB from 7 until 35 minutes post-fertilization, blocking both the first and second polar body division, which resulted in 24.1% - 2N, 58.7% - 3N and 17.2% - 4N spat. In autumn 2004, 4N induction trials were attempted at the Bedford Institute of Oceanography utilizing methodology proposed by Scarpa *et al.* (1993). This may be the next step in producing commercial numbers of 3N mussels without the necessity of handling potentially harmful induction chemicals (i.e., CB). While human exposure to CB in concentrations used to induce triploidy are potentially harmful (Sigma 2002-2003), studies have yet to be designed to assess the persistence of CB in market-sized shellfish.

Davis (1997) has modified chemical induction techniques developed by Desrosiers (1993) which involves treating fertilized *M. galloprovincialis* eggs with a combination of heat shock (5-10 °C above ambient) and 6-DMAP (30-300 µM) during the period corresponding with 2nd polar body release. According to Davis, this results in nearly 100% 3N larvae and juveniles. This method can now be used to treat commercial amounts of *M. galloprovincialis* eggs (100-300 million). Similar to Kiyomoto *et al.* (1996), field trials conducted by Davis have revealed that 3N's still undergo limited gametogenesis; as well, there are no apparent differences in growth rates and survival despite exposure to varying environmental conditions; however, experiments conducted by Brake *et al.* (2004) revealed that 3N *M. edulis* grow faster than 2N siblings in high growth sites during both the 1st and the 2nd year of growth, most likely due to a reallocation of glycogen reserves normally reserved for gonadal development in 2N's. Gordon King, the Manager of the Mussel Department of Taylor Resources Inc., Washington State, U.S.A. (pers.comm. 2004), has said that while there are not major differences in growth, 3N *M. galloprovincialis* tend to grow slightly slower than 2N *M. galloprovincialis* in their first year of growth and when reared in areas of limited food availability. Brake *et al.* (2004) has since confirmed this by showing that while 2N and 3N *M. edulis* full siblings do not significantly differ in growth rate during the 1st year when grown in low growth sites, 3N's were significantly ($P<0.001$) longer (10.95%) and possessed a higher condition index (30.62%) than 2N's after a second year of growth. Nonetheless, as evidenced by an only 28% incidence of 3N's (down from ~80% 3N incidence at deployment) after 23 months, 3N's grown under less favourable conditions appeared to die disproportionately to 2N's grown under similar conditions. In Pacific oysters there is site-specific field evidence to suggest that chemically induced 3N's actually lose chromosomes over time, accumulating more and more 2N cells, thus reverting to a heteroploid mosaic state (Allen *et al.* 1999). Nonetheless, it is unlikely that the dramatic decrease in 3N incidence seen by Brake *et al.* (2004) at the low growth site was due entirely to reversion from the 3N to 2N state because 3N reversion, at least in Pacific oysters, usually results in heteroploid mosaic animals that are easily identified by current flow cytometric techniques (Allen *et al.* 1999).

Despite the apparent decrease in 3N survivability for *M. edulis* when grown in unfavourable environments, 3N production accounted for nearly 20% of Taylor Resources Inc. total *M. galloprovincialis* production in 2003, and acts to fill an economic void left by the deterioration of 2N mussel quality and appearance during their fall spawning season. Thus, besides the potential benefits of 3N's for introduction and transfer into ecologically sensitive areas, there appears to be a considerable niche (20% of total production) for the commercial production of 3N *M. galloprovincialis* on the Pacific coast of North America. This niche is yet to be produced for *M. edulis* but is of mounting interest to commercial producers in the Maritime Provinces of Canada (Brake *et al.* 2004; Gordon King, pers.comm. 2004).

TRIPLOIDS: IN SUMMARY

Although useful for introductions/transfers, more work examining the benefits of 3N's for commercial culture in Atlantic Canada must be conducted. We are currently running preliminary experiments designed to measure the growth of 2N's and 3N's under laboratory conditions. Although, Davis's (1997) experiences with *M. galloprovincialis* revealed no significant differences in growth and survivability between 2N's and 3N's, recently published literature (Brake *et al.* 2004) and laboratory experiences at BIO might suggest otherwise for *M. edulis*.

MATERIALS AND METHODS

CYTOCHALASIN B TRIPLOID INDUCTION

Spawning

One *M. edulis* male (Identification code: 2000WM2) and 3 ripe pedigreed female-biased *M. edulis* female broodstock (Identification codes: X102Q, X102R and 99WF7) (Kenchington *et al.* 2002) were utilized for 3N induction. Both X102 individuals (Q and R) were full siblings produced from a cross between female-biased female X and male 102, while female-biased female 99WF7 was a wild female collected and first spawned in 1999. On November 25th 2003, broodstock were removed from a tank containing ~8°C unfiltered seawater pumped from a depth of ~20 m, scrubbed with a fine bristle brush using domestic fresh water, wrapped in damp paper towel and refrigerated for ~18 hrs at 4°C. The following day, refrigerated broodstock were removed and placed into individually labelled 500 ml dishes filled with 20°C UV treated, 1µm filtered seawater to induce spawning. Post-spawn, broodstock were removed and sperm/eggs were collected individually and filtered through 60 µm Nitex mesh to remove feces.

Triploidy Induction

Roughly half of all eggs procured from each of the 3 females were allocated for chemical 3N induction. The other half was used to produce 2N controls for each female. Eggs intended for 3N induction were concentrated into individual 1 L beakers filled with 250 ml of UV treated, 1 µm filtered 14°C seawater and fertilized with sperm at a concentration of 10-15 sperm/egg. Starting 10 min post-activation and every 5 min thereafter, a small sample of fertilized eggs was examined microscopically to establish the point at which ~50% of all fertilized eggs had released the first polar body. Based on values suggested by Beaumont and Kelly (1989) and Jackson *et al.* (2003), ~25 min post-fertilization eggs from both 99WF7 and X102R were treated with 125 µl of CB (1 mg/ml CB in DMSO) in 250 ml of seawater to achieve a final CB concentration of 0.5 mg/l and 0.05% DMSO. However, fertilized eggs from X102Q were treated with a working CB concentration of 0.8 mg/l (200 µl (1 mg/ml CB in DMSO)/250 ml). The fertilized eggs in the CB solutions were regularly plunged every 1-2 min to homogeneously expose eggs to CB over the 15 min treatment duration. Post-treatment, early embryos were gently poured onto a 20 µm Nitex screen and rinsed for 2-3 minutes with UV treated 14°C, 1 µm filtered seawater. They were then treated with 0.02% DMSO in seawater for 15 minutes to remove any persisting traces of CB (Jackson *et al.* 2003).

Both 2N controls and 3N – CB treatment groups were counted and split evenly into 6 buckets, one for each female/ploidy combination, at a density of 50 eggs/ml (300,000/5L bucket). Approximately 14,000,000 remaining eggs spawned by X102Q and X102R were also induced using the same methods and concentrations as stated above (0.5 mg/L CB and 0.05% DMSO). Post-CB treatment, the developing zygotes from these females were placed into a 250 L tank topped with UV treated 14°C, 1 µm filtered seawater.

Larval Rearing

On November 29th, 2003 (day 4) survival and mean shell length (µm) (N=20) were assessed and recorded from each of the 2N control and 3N – CB treatment buckets. Mussels from both 2N and 3N 5 L buckets were thinned to a density of 10 larvae/ml. On December 1st, the stocking density of mussels in the 250 L CB treatment tank was assessed at approximately 15 larvae/ml. Initial survival rates were determined for mussels from each female/ploidy combination on December 9th, 2003. On this date it was discovered that nearly all mussels in the 250 L tank were dead or dying. The smallest, mostly dead, larvae were extracted by pouring larvae over an 80 µm mesh. Remaining larvae >80 µm from the 250-L CB-treatment tank were placed into a 5-L bucket at a density of ~4/ml or 20,000/bucket.

Water from 2N control and 3N – CB treatment buckets was changed daily using Nitex screen of ascending mesh size as mussel size increased. Animals were fed three to four times/day with a mixture of flagellates (i.e. *Isochrysis galbana*, *Pavlova pinguis* and *Tetraselmis sp.*) and diatoms (i.e. *Chaetoceros calcitrans*, *Chaetoceros mulleri*, *Skeletonema costatum* and *Thallassiosira pseudonana*) depending on their availability. Food consumption was monitored visually by assessing water clarity on a daily basis. In an attempt to avoid limited growth in response to increasing biomass, animals were thinned by increasing bucket numbers and splitting treatments. Finally, on January 26th, 2004 (2 months post-spawn) 210 diploids and 210 triploids produced from each female (X102Q, X102R and 99WF7) were placed into 2 separate 10 L buckets for each female (a total of 6 buckets). The remaining 2N and 3N mussels from each female were pooled and placed into 2 separate 250 L tanks (2N and 3N) filled with UV treated 14°C, 1 µm filtered seawater at ~12,000-15,000 spat/tank.

Flow Cytometric Triploid Induction Rate Assessment

On November 28th (day 4), December 9th (day 14), December 23rd (day 28) and January 2nd (day 38), several hundred larvae and/or early spat were collected in 1.7 ml microcentrifuge tubes from both 2N control and 3N – CB treatment buckets from each female (X102Q, R and 99WF7) cross. Mussel samples were also taken from a 5 L bucket remaining from the initial 250 L tank which contained 3N mussels from females X102R and X102Q. Following detailed protocols for flow cytometric sample preparations provided by Jackson *et al.* (2003), supernatant seawater was removed from 1.7 ml microcentrifuge sample tubes and was replaced with 0.5 ml of prepared Propidium Iodide solution. Tubes were then mixed on a Fisher Vortex Genie 2 for ~20 seconds. After incubating in the dark at room temperature (~20°C for 10 minutes), samples were mixed for ~10 more seconds prior to being filtered through a Becton Dickson 20 µm syringe Filcon to remove any large chunks of tissue or shell before running on the Becton Dickson FACSCalibur flow cytometer. In a similar procedure performed on February 4th (day 71), 3 vials of 10 mussels from both the 2N control and 3N – CB treatment buckets were prepared and run on the FACSCalibur to confirm previously acquired 3N induction rates. The difference being, these larger mussels (2-3 mm) were crushed prior to the addition of propidium iodide via the Becton Dickinson Medimachine System

(Mississauga, Ontario), a sample preparation system for the disaggregation of solid animal tissues, to allow for maximum nuclear staining.

Data was initially collected and analysed using CELL Quest Pro software (Becton Dickson and Company) prior to further scrutiny via ModFit LT Software (Verity Software House, Inc.). ModFit LT has the capacity to differentiate and normalize 2N and 3N peaks and to more accurately describe 3N induction success.

Preliminary Diploid and Triploid Growth Experiment

On January 8th, 2004 (day 44), 200 mussels from the 2N control buckets and 200 mussels from the 3N – CB treatment buckets, produced by the female 99WF7, were collected. Only offspring from 99WF7 were chosen for this experiment because this cross exhibited elevated survival prior to day 44 as compared to the other females and space was limited. Fifty of the 200 collected mussels from both the control and CB treatment buckets were measured for shell length (µm). Two hundred mussels were then distributed to 2 – 5 L buckets of 2N's at 100/bucket. This process was repeated to produce 2 – 5 L buckets of 3N mussels at 100 mussels/bucket. Seawater from all buckets was changed daily and kept between 17 and 18.5°C. Mussels were fed with increasing quantities of both flagellates and diatoms as clearing rates escalated do to the mussels increasing size. Shell length was initially measured weekly starting at day 1 of the experiment and visual clearing rates were noted for each bucket over the duration of the experiment. Feed, bucket number and biomass was adjusted accordingly for each treatment depending on the growth and algal clearing rates observed over the experimental time period. A more detailed description of the feeding, husbandry and sampling regimes for this preliminary experiment is provided in Appendix 2.

HEAT SHOCK TRIPLOID INDUCTION

Spawning and Triploid Induction

On May 26th, 2004 1 female (*M. edulis* x *M. trossolus)* hybrid and 5 male *M. edulis* broodstock taken from a holding tank (~1.1°C unfiltered seawater pumped from a depth of ~20 m) were induced to spawn in individual 500 ml dishes by submersion in 25°C UV treated 1 µm filtered seawater. Post-spawn, broodstock were removed and sperm/eggs were collected individually and filtered through 60 µm Nitex mesh to remove feces. Eggs were concentrated in a 1 L glass beaker at ~1490 eggs/ml (1.49×10^6 eggs total) and held at 20°C for ~30 minutes prior to being fertilized with pooled sperm (5 males) at a concentration of ~12 sperm/egg. Starting 10 min post-activation and every 5 min thereafter, a small sample of fertilized eggs was examined microscopically to establish the point at which ~50% of all fertilized eggs had released the first polar body. At ~20 minutes post fertilization, eggs were poured onto a 20 µm Nitex screen and submerged into a UV, 1 µm filtered, 30.8°C water bath for 10 minutes. An equivalent amount of fertilized eggs from the same female were fertilized with pooled sperm and used as a 2N control. Fertilized eggs from both the 2N and 3N groups were placed into individual 5L buckets at 300 eggs/ml. On day 5 (May 31st, 2004), there was a large

undiagnosed mortality event in both the 2N control bucket and 3N heat shock treatment bucket. Nonetheless, for both the 2N control bucket and the 3N heat shock treatment bucket the remaining larvae were reduced to 10 and 3/ml respectively on day 7. On June 7th (day 12), all mussel D-larvae in both buckets were discovered dead and thus the experiment could not be continued.

Flow Cytometric Triploid Induction Rate Assessment

On May 28th, 2004 hundreds of early larvae were collected from both the 2N control and 3N heat shock treatment buckets for flow cytometric ploidy analysis. Triploid induction success was assessed via identical protocols utilized in the CB 3N induction procedures.

RESULTS

CYTOCHALSIN B TRIPLOID INDUCTION

Post Spawn

Five days post-spawn, 2N control and 3N CB-treated 5-L mussel buckets for each female cross (a total of 6 buckets) were reduced in stocking density to 10 larvae/ml or 50000 larvae/bucket. Population estimates calculated twice over a 5 day period from day 7 to day 12 post-spawn revealed a slight decrease in density for both 2N's and 3N's (~13.5% and 16.1%) respectively. However, there did not appear to be any significant differences in survival between 2N controls and 3N CB-treated mussels over this time period.

On December 10th, 2003 all 2N control and 3N CB-treatment buckets and the 250-L tank containing ~3.75 x 10^6 CB treated larvae from females X102R and Q were to be reduced in stocking density to 4/ml. Unfortunately, it was discovered that a large proportion (> 95%) of CB treated larvae in the 250 L tank were dead or dying from an unknown cause.

Triploidy Induction Rate

Ploidy confirmation and induction success rates for 2N control and 3N CB-treated groups are recorded in Table 1. Regardless of which female's eggs were induced, treating fertilized eggs with 0.5 to 0.8 mg/l CB resulted in a 3N induction rate of nearly 100% (95 to 100%) as indicated by sub-samples of larvae and spat taken for ploidy analysis from each bucket from 2 to 37 days post-spawn. As well, 2N control groups were confirmed nearly 100% 2N's for all samples tested; however, some stained 3N nuclei were captured in samples tested from X102R control on days 2, 13 and 27 post-spawn.

As shown in Table 1, ploidy confirmation of larvae from the 250 L tank which contained the remaining CB treated eggs from females X102R and X102Q revealed a nearly 100% (97.70 ± 1.80) 3N induction at 2 days post-spawn. However, at day 27 post-spawn or 14 days after a large mortality event where nearly 95% of the entire population died, ploidy analysis via flow cytometry revealed that the proportion of 3N's had decreased by nearly 37%. Figures 2 and 3 contain ModFit LT graphics and statistics that clearly display this change in 2N and 3N ratio.

Table 1: Summary of blue mussel triploid (3N) induction rates achieved during November 2003 spawn at the Bedford Institute of Oceanography.

Treatment	Female	Bucket	Days Post-Spawn	Sample (#)	Ploidy (%)	
					2N/Aneuploid	3N
0.5mg/l CB	X102R	A	2	1	0.00	100.00
		B		1	0.87	99.13
		A	13	1	0.05	99.95
		A	27	1	4.60	95.40
		A	37	1	2.56	97.44
Control		A	2	1	100.00	0.00
		B		1	98.53	1.47
		A	13	1	97.47	2.53
		A	27	1	99.09	0.91
		A	37	1	100.00	0.00
0.8mg/l CB	X102Q	A	2	1	0.08	99.92
		B		1	0.19	99.81
		A	13	1	0.10	99.90
		A	37	1	0.00	100.00
Control		A	2	1	100.00	0.00
		B		1	100.00	0.00
		A	13	1	100.00	0.00
		A	37	1	100.00	0.00
0.5mg/l CB	99WF7	A	2	1	0.08	99.92
			13	1	0.03	99.97
			27	1	1.49	98.51
			37	1	0.00	100.00
Control		A	2	1	100.00	0.00
		B		1	100.00	0.00
		A	13	1	100.00	0.00
		A	37	1	100.00	0.00
0.5mg/l CB	X102Q/ X102R	250 L Tank	2	3	2.20 ± 1.80	97.70 ± 1.80 *
			27	1	38.20	61.80

* Note: Samples of typical ModFit LT cell cycle analysis data, graphics and statistics provided in Figures 1 and 2.

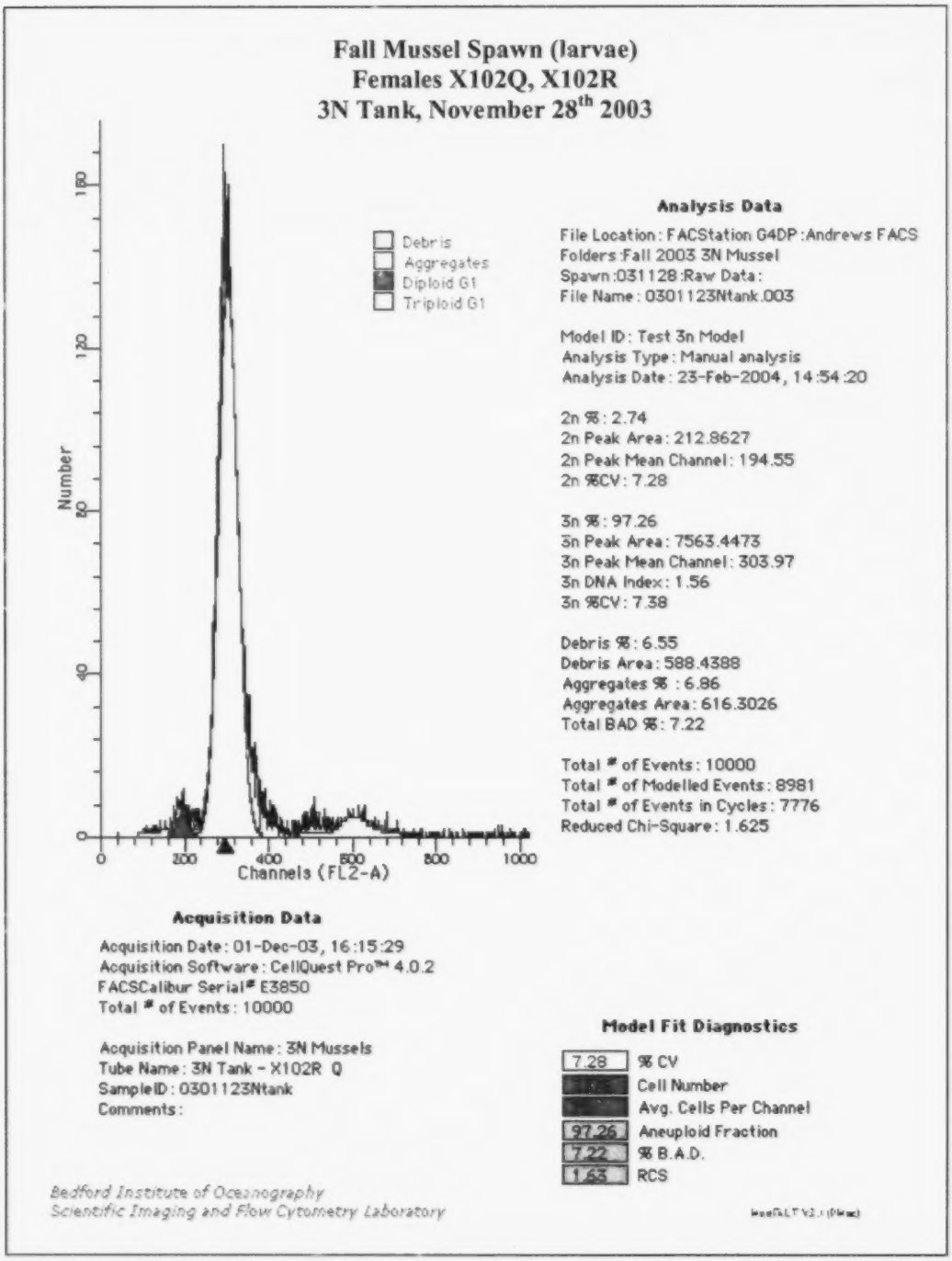

Figure 1. Typical ModFit LT output of 3N induction success observed for each female cross utilizing a 0.5 mg/l CB concentration.

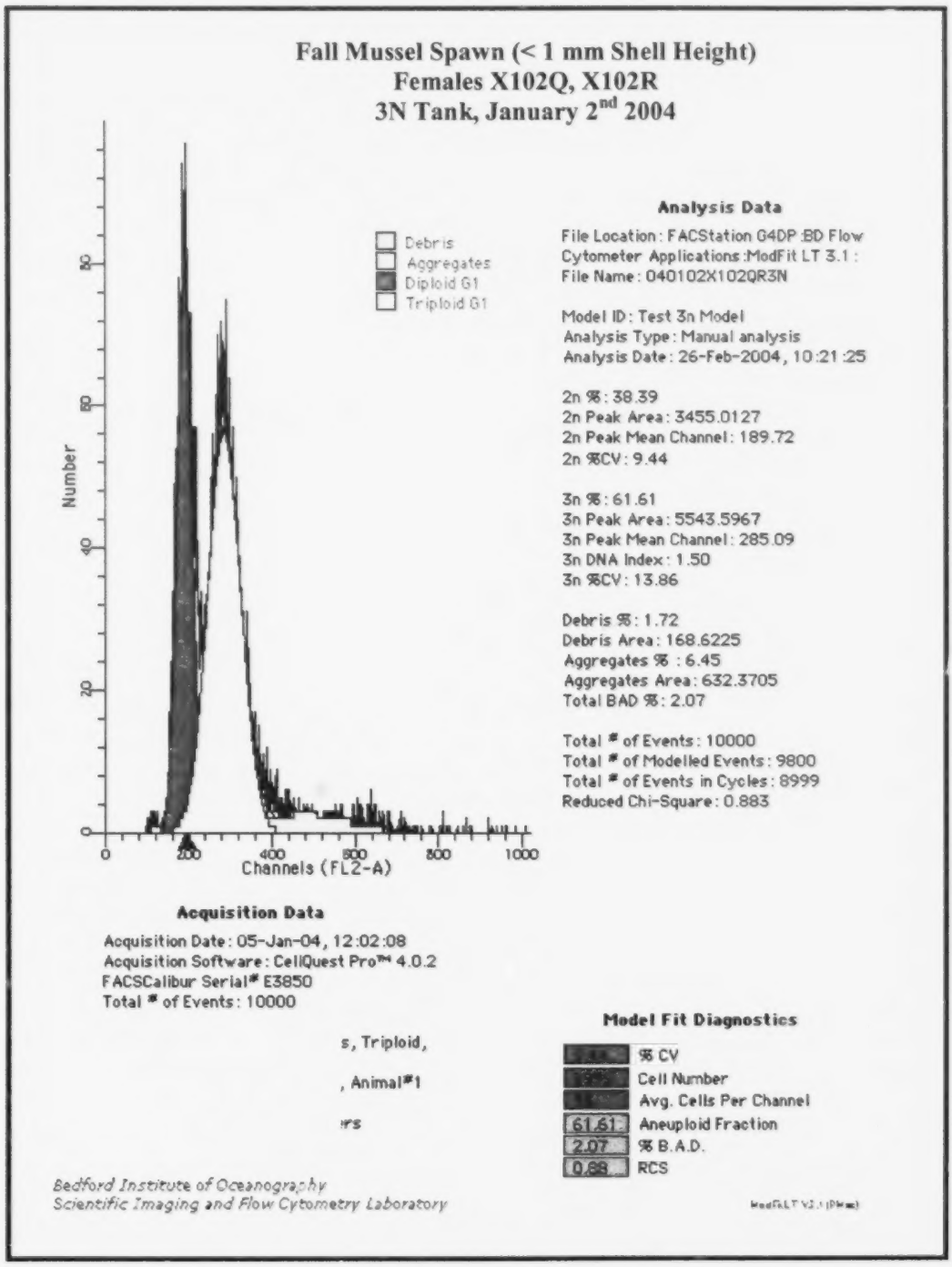

Figure 2. Ploidy distribution of CB treated mussels from females X102R and X102Q observed 14 days after a large mortality event where nearly 95% of the entire population died. Cytometric analysis revealed a 37% decrease in the abundance of 3N's.

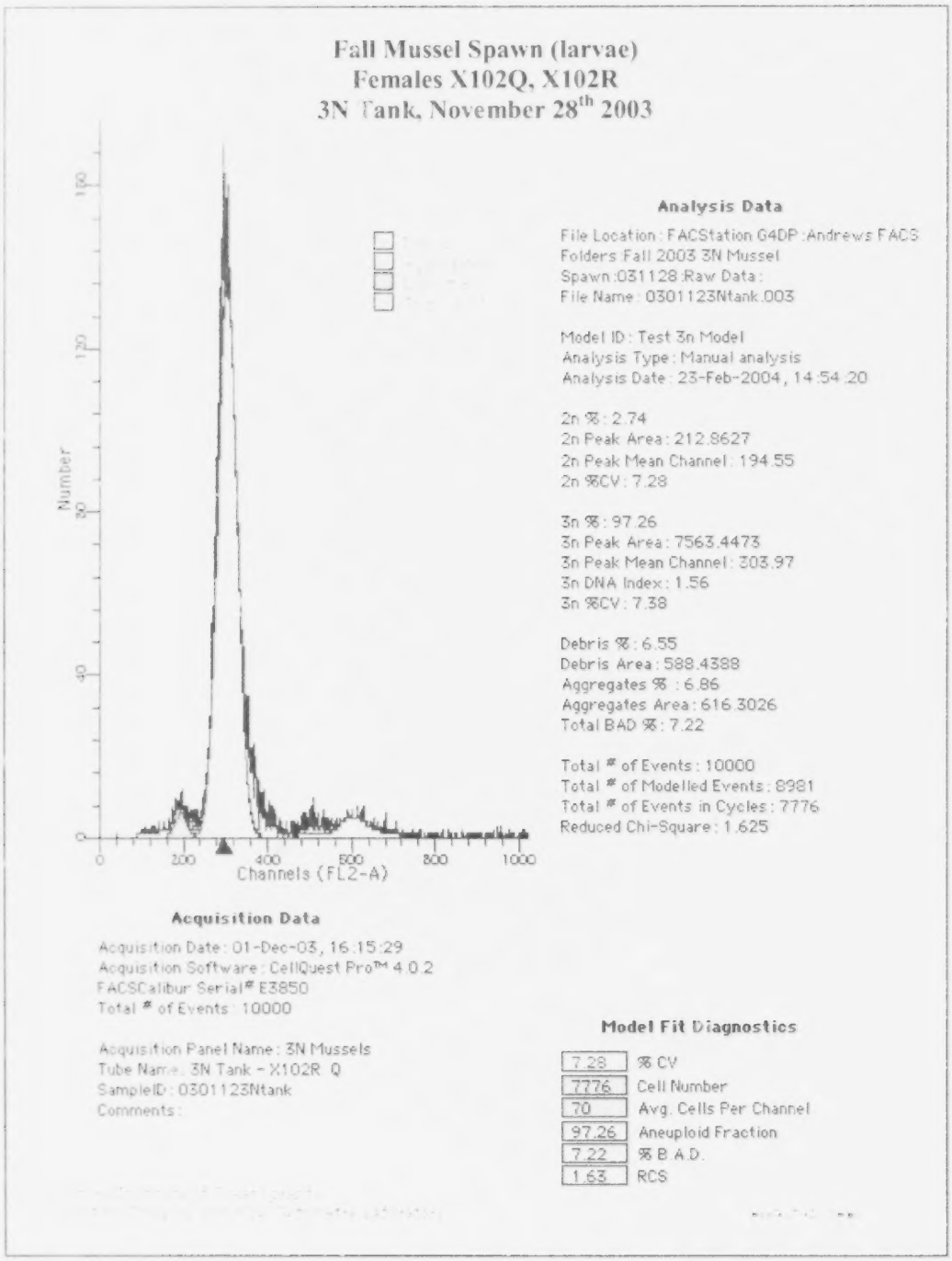

Figure 1. Typical ModFit LT output of 3N induction success observed for each female cross utilizing a 0.5 mg/l CB concentration.

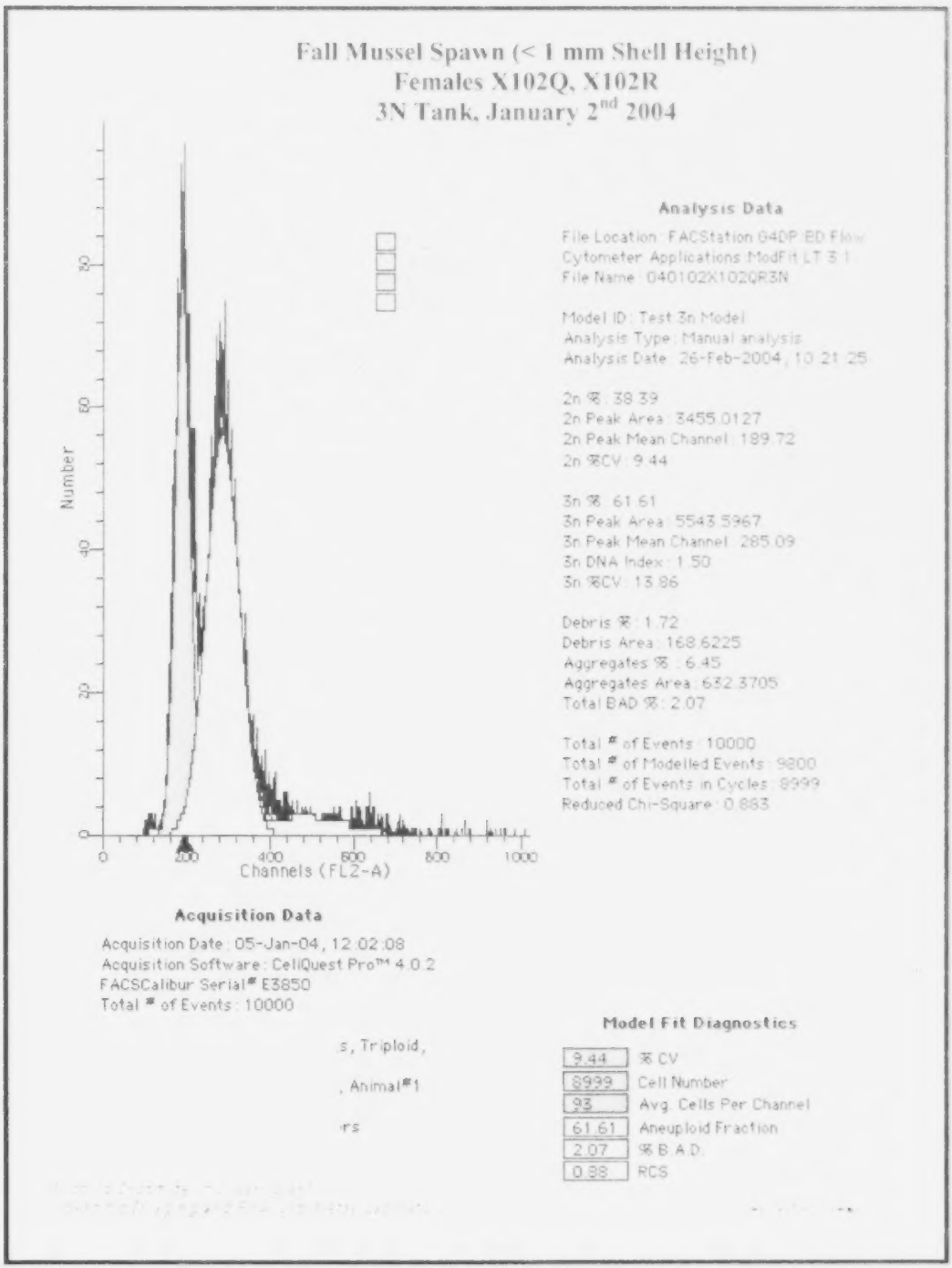

Figure 2. Ploidy distribution of CB treated mussels from females X102R and X102Q observed 14 days after a large mortality event where nearly 95% of the entire population died. Cytometric analysis revealed a 37% decrease in the abundance of 3N's.

Preliminary Diploid And Triploid Growth Experiment

There were no dramatic differences in growth between 2N and 3N mussel spat, as evidenced by shell length, shell height, and shell width over the duration of these observations. As shown in Figures 3, 4 and 5 when grown under identical culture conditions (feed, temperature and density) both 2N and 3N blue mussels in all groups grew at nearly identical rates. While there were never significant differences in shell height observed between the two 2N groups (2N A and 2N B) or between the two 3N groups (3N A and 3N B), significant differences in shell length as determined by 2 tailed paired t-tests ($P<0.05$) were observed for the first 6 sampling dates between cumulative shell length data collected from spat/juveniles from the 2N buckets (2N A and 2N B) and the 3N buckets (3N A and 3N B). The difference was small however, and remained constant over the first six weeks of the experiment, with the mean 2N shell height being consistently 1% (1.08 ± 0.05 %) greater than mean 3N shell height at each sampling point.

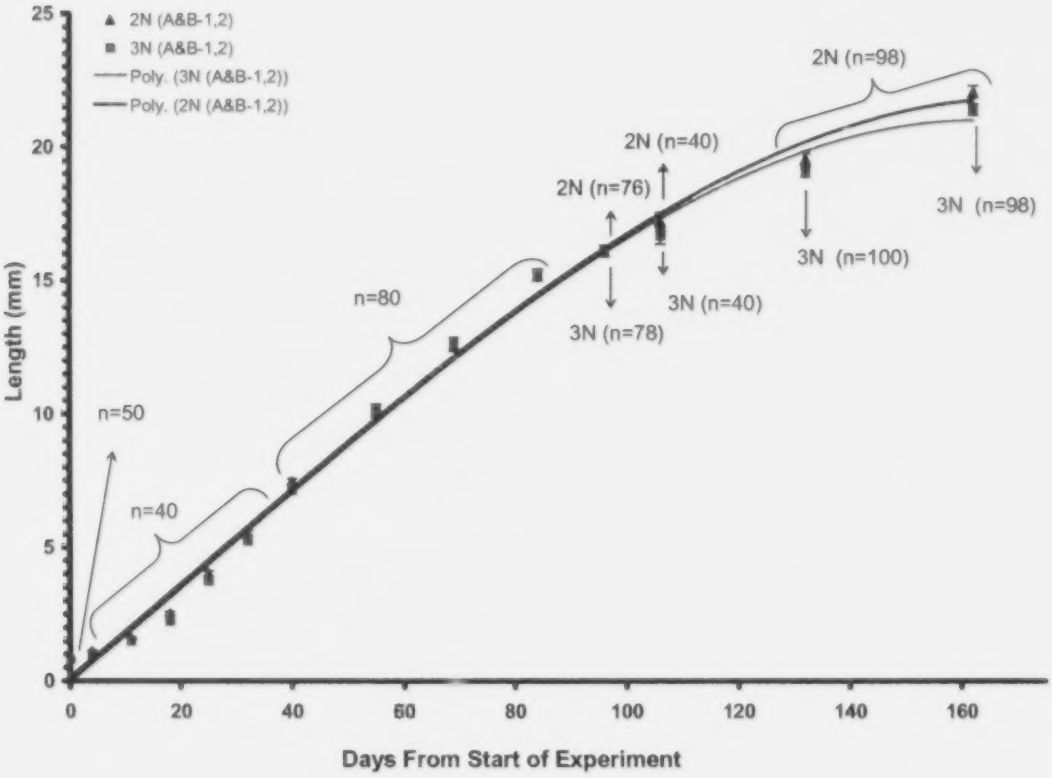

Figure 3. Diploid (2N) and triploid (3N) spat and juvenile mean shell length over 23 weeks produced from female 99WF7 (mean length ± standard error).

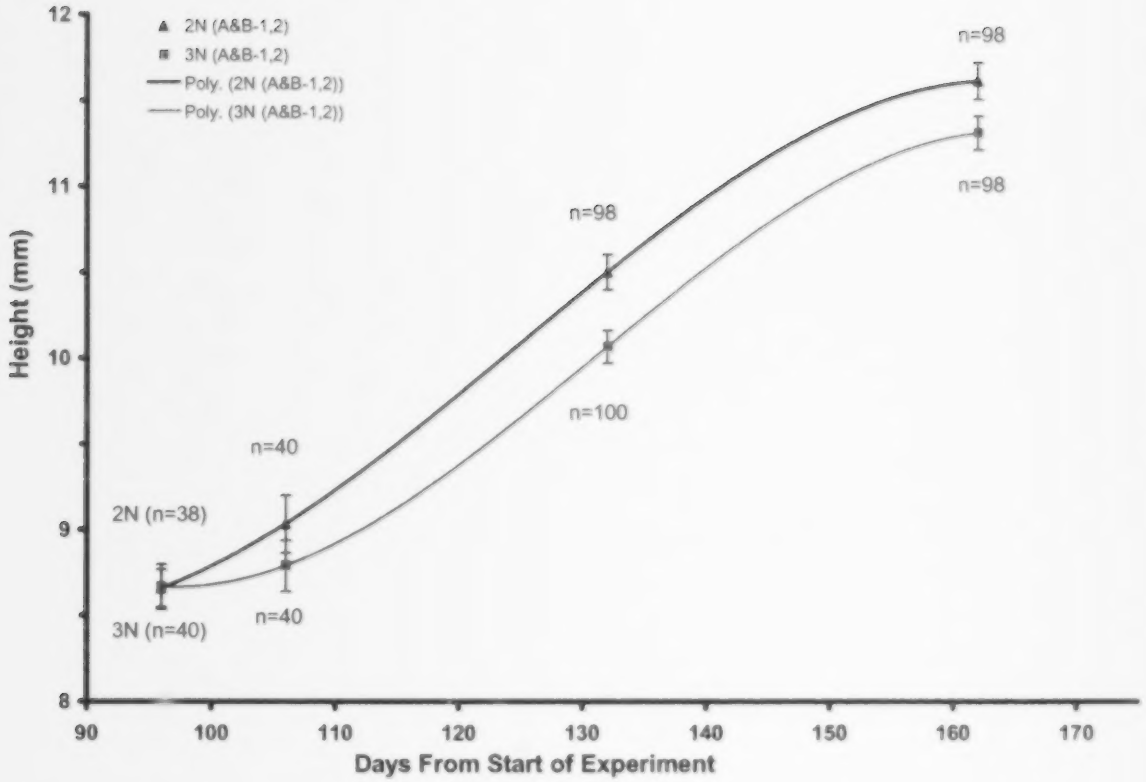

Figure 4. Diploid (2N) and triploid (3N) juvenile mean shell height from week 14 to week 23 produced from female 99WF7 (mean height ± standard error).

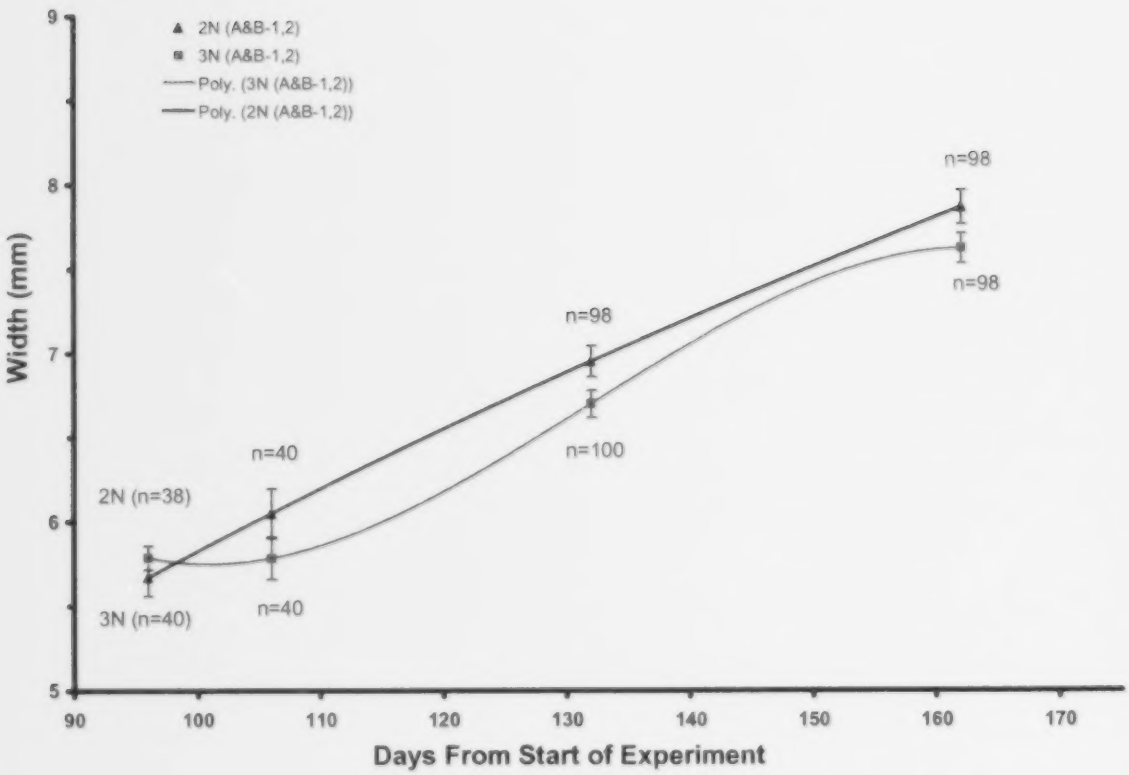

Figure 5. Diploid (2N) and triploid (3N) juvenile mean shell width from weeks 14 to 23 produced from female 99WF7 (mean width ± standard error).

In conjunction with growth parameters, it was also noted that water in the 2N control buckets was visibly cleared of color after feeding at a faster rate than in the 3N CB-treatment buckets. Although these were only visually observed differences, they were consistent and obvious enough over the entire experimental duration that it has prompted further interest in exploring the underlying causes responsible for this decline in filtration rate for 3N blue mussels.

Based on the results observed, more complex growth experiments examining 2N and 3N metabolic rates are currently being developed. It is our goal to continue growth experiments beyond the lab and into the field for 2005-2006.

HEAT SHOCK TRIPLOIDY INDUCTION

Post Spawn

The main intention of this preliminary heat shock trial was to test the 3N induction power of previously described heat shock methods (Yamamoto and Sugawara 1988). Nonetheless, early survival data collected on May 28th, 2004 revealed that after 48 hours 3N survival to D-larvae was only 6.7% of the beginning total of 1.49 x 10^6 larvae, while 2N D-larvae were at nearly 47% of their initial egg stocking density. While the rate of survival of 2N controls was ~20% less than that seen for 2N controls in the CB experiments (~67% survival at day 2), heat shock 3N survival at day 2 was ~62% less than that seen for CB induced triploids at day 2 (~68% survival at day 2). This was early evidence that the heat shock method results in extremely high mortality rates in comparison to the CB induction method. Unfortunately, no further survival data could be collected because on day 5 an undiagnosed mortality event claimed nearly every mussel from both the 2N and 3N groups.

Triploidy Induction Rate

Early 3N induction rate assessment on May 26th, 2004 via ModFit LT revealed a ~69% induction rate (Figure 6). Based on these positive early results, further heat shock trials will be attempted in 2005.

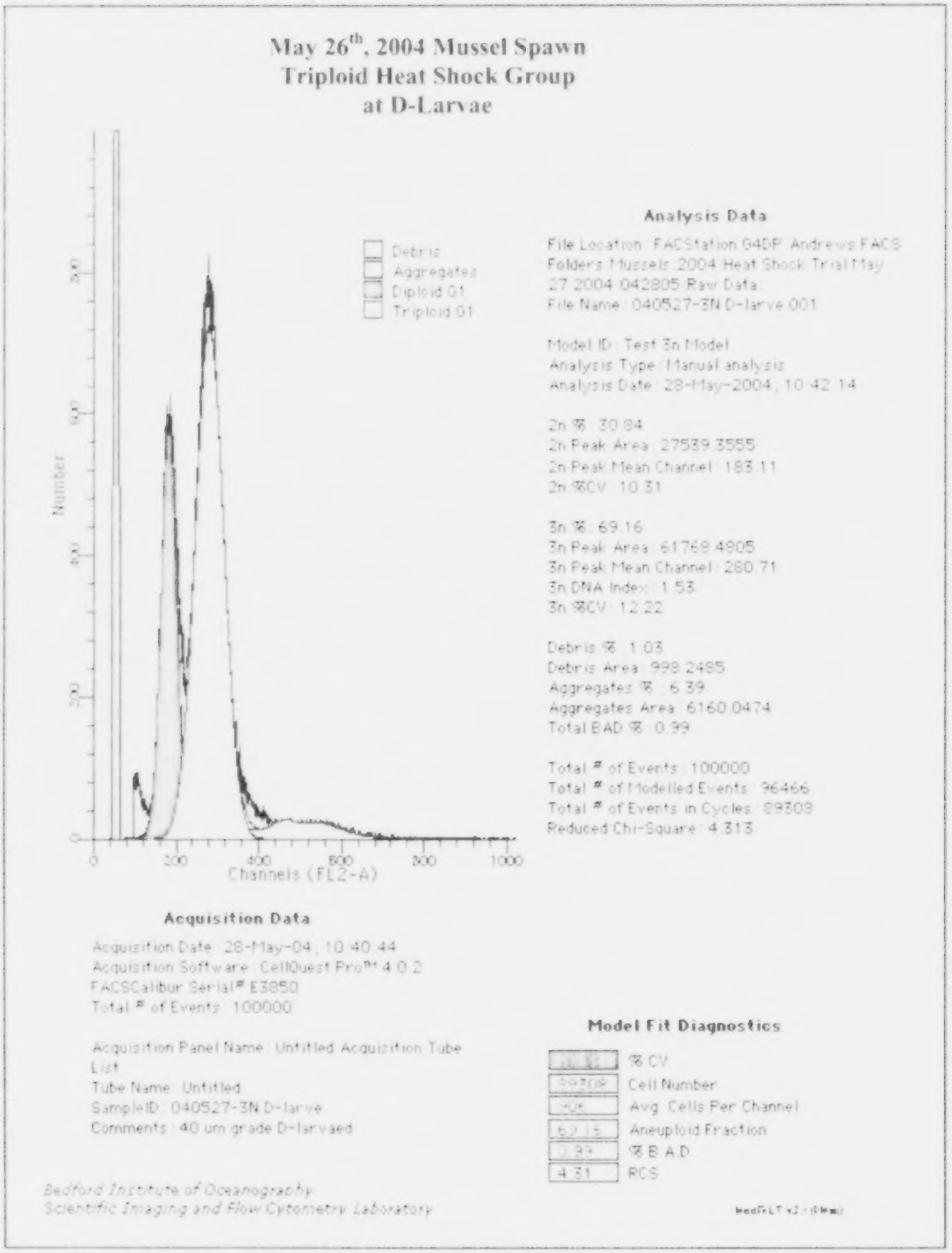

Figure 6. ModFit LT output of triploid (3N) heat shock induction success observed on May 26th, 2004 (30.8°C for 10 minutes, 20 minutes post-fertilization).

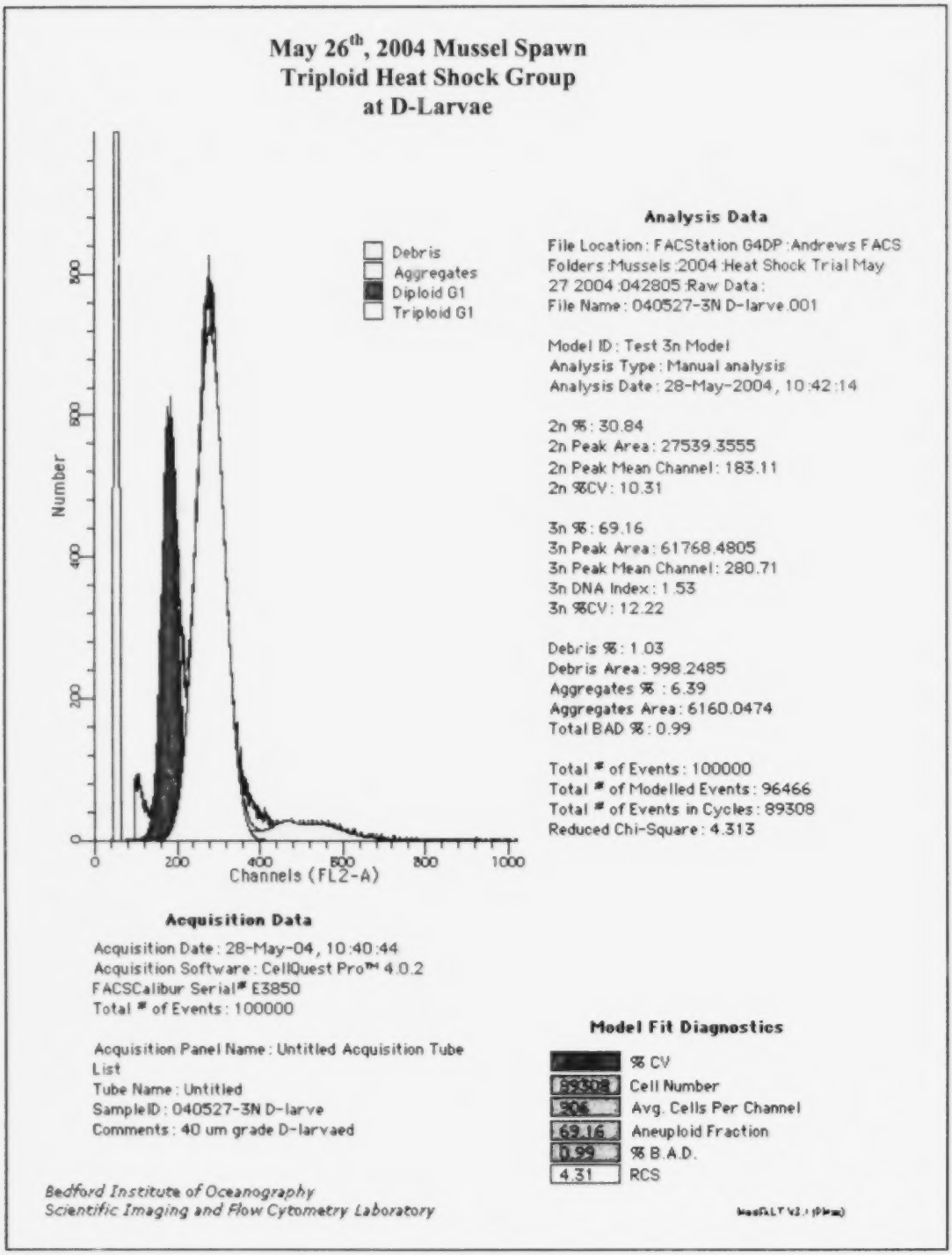

Figure 6. ModFit LT output of triploid (3N) heat shock induction success observed on May 26th, 2004 (30.8°C for 10 minutes, 20 minutes post-fertilization).

DISCUSSION

POST SPAWN

There were no significant differences in shell length, height, width and wet weight observed between 2N and 3N *M. edulis* through 6 months of growth under laboratory conditions. In addition, our results revealed that while survival to D-stage was reduced in 3N treatment groups as compared to 2N controls, there was no significant difference in survival between controls and CB-treatment groups post metamorphosis under laboratory conditions. This being said, it did appear as though after a mortality event on December 9th, 2004 (27 days post spawn) that killed nearly 95% of the entire population of nearly 100% 3N D-larvae from a 250 L tank which contained the remaining CB treated eggs from females X102R and X102Q, that 3N's were more susceptible to mortality when reared in water of poor quality, or in the presence of a pathogen.

PRELIMINARY DIPLOID AND TRIPLOID GROWTH AND SURVIVAL

The phenomenon of increased growth for 3N's has been observed in many species of commercially important shellfish and has been largely attributed to, among other factors, the reallocation of glycogen reserves to somatic tissue at a time when 2N's are storing these reserves for gonadic maturation (Allen and Downing 1986). As is seen in some species 3N individuals show improved growth before the onset of maturation, this is not always the only cause for improved growth (Guo and Allen 1994a). As well, increased heterozygosity among 3N's due to an additional set of chromosomes seems to be a contributing factor to their increased growth. There is evidence to suggest that increasing degrees of heterozygosity in 3N's (as evidenced by polar body II, polar body I and tetraploid x diploid matings) results in a positive correlational increase in overall meat weight (Wang *et al.* 2002). Lastly, similar to what has been seen in Atlantic salmon (*Salmo salar*), triploidy results in larger overall cell size to accommodate their elevated nuclear volume (Benfey and Sutterlin 1984). This has not resulted however in larger salmon because unlike shellfish, finfish posses a homeostatic mechanism which reduces cell number thus resulting in a 3N animal of similar proportions to their 2N counterparts. In shellfish (notably clams – (Guo and Allen 1994)), this regulative mechanism does not appear to exist and the resulting increase in cell size from 3N induction sometimes produces inherently larger animals. It is likely that not just any one of these factors (energy reallocation, heterozygosity and gigantism) contributes solely to the elevated growth rates observed in various species of shellfish but that each factor contributes in varying degrees in accordance with environmental factors and species specific developmental stages (Beaumont and Fairbrother 1991).

While shell morphometric data from this experiment did not significantly differ between 2N and 3N groups, there was visual evidence at least that 3N's, even when stocked at identical densities to 2N's, took longer to clear the water of algae after feeding. Feeding rates and 3N physiology were not the primary area of interest for this study; however, the lack of significant differences between the various shell parameters combined with observable differences in feeding rate and preferential 3N mortality under

less than optimal conditions has spurred research interest directed at the cause of both of these effects. Mr. Gordon King (pers.comm.) suggested that there is some anecdotal evidence that 3N *M. galloprovincialis* do not grow well under poor field conditions. Again, while 3N mussel vigour under poor growing conditions or in the presence of a pathogen has not yet been formally examined, evidence from Brake *et al.* (2004) and this report, suggests that 3N's exhibit preferentially reduced survival as compared to 2N's when grown in poor conditions. While these observations are unquestionably subjective this does not negate both the importance and need for future scientific evaluation of these observations. Future work examining both growth and survival of 3N and 2N mussels while purposely varying abiotic and biotic conditions in the laboratory (e.g., water temperature, feeding level/type, and stocking density) and in the field (e.g., wave action, exposure to pathogens and grow out densities) should be conducted before the commercial applicability of chemically induced (CB) 3N blue mussels can be properly assessed.

No work has been done examining metabolic scope in 3N mussels; however, some work examining the bioenergetics in Pacific oysters has revealed that 3N's spend ~26% more energy for growth than 2N's and 13% more for assimilation (Zhou *et al.* 2000). Observations from this report seem to suggest a slight decrease in filtration rate either because 3N mussels are metabolically more efficient and need less food to achieve optimum growth as compared to 2N's or because their filtration mechanism is somehow deficient. This second option would seem to suggest then, that 3N's would grow equally as well as 2N's in favourable environments, but would not grow as well in unfavourable environments. It is possible that in high food environments both the 2N's and 3N's meet metabolic requirements (regardless of any filtration mechanism problems) but the 2N's filter their food more efficiently. Thus it will be necessary not only to examine 2N and 3N metabolism, but to measure it under varying feeding regimes.

As mentioned in the Introduction, Brake *et al.* (2004) did observe significant increases in numerous growth parameters (shell length, condition index, etc.) for 3N's in both low and high productivity sites, but no mention was made of early growth for pre-deployed hatchery 2N and 3N *M. edulis*. Our results tracking growth of larvae and spat prior to deployment for grow-out supports the belief that the increased growth in 3N's observed by Brake *et al.* (2004) is unlikely due to polyploidy gigantism (because of no observed increase in growth- rate prior to the onset of sexual maturation) but is mostly a product of energy reallocation during developmental periods associated with gonadal development.

TRIPLOIDY INDUCTION RATE

At 95-100%, CB-3N induction rates reported here are as high as or higher than previous trials utilizing CB or any other form of induction with *M. edulis*. Triploidy inductions utilizing a final concentration of 0.5 mg/l CB resulted in induction rates that were ~11-30% higher than previous reports utilizing similar methods (Scarpa *et al.* 1994; Beaumont and Kelly 1989). Nonetheless, there have been positive results reported utilizing other methods. For example, heat shock has been used effectively, but

erratically, by numerous researchers to produce triploids (25-97%) (Scarpa *et al.* 1994; Beaumont and Kelly 1989; Yamamoto and Sugawara, 1988). Observed differences in 3N induction via heat shock has been attributed to experimental error (Yamamoto and Sugawara 1988), as evidenced by a small portion of 3N's in their 2N controls (2.5-22%) (Brake *et al.* 2004; Beaumont and Kelly (1989)), Scarpa *et al.* (1994) also observed this phenomenon in their control groups. The heat shock method should be re-evaluated with exposure timing based on the observation of 50% polar body release (Jackson *et al.* 2003) rather than an arbitrary time (10, 20, 30 or 40 minutes) that might be altered depending on the pre-fertilization egg incubation temperature. A single heat shock trial carried out at the Bedford Institute of Oceanography involved immersing fertilized eggs at 50% 1st polar body release into 30.8 °C seawater for 10 minutes. Ploidy tests after 48 hours revealed that 3N induction was ~70%; a value within 5% of that shown by Yamamoto and Sugawara (1988) when using a similar heat shock temperature (30°C). While induction rates utilizing this method are reduced compared to chemical treatment with CB, further modification may improve the efficiency of this method.

Downing and Allen (1987) pointed out that there are four major factors that influence the effectiveness of CB when inducing triploidy: 1) dosage, 2) duration of treatment, 3) treatment temperature, and 4) time after fertilization when the treatment is applied. The increase in induction rate seen in our trials is likely accounted for by the timing of CB exposure after fertilization. In studies by Scarpa *et al.* (1994) and Beaumont and Kelly (1989) the timing of 3N induction, via the retention of the second polar body of Meiosis II, was based on time after fertilization (20 minutes and 30 minutes respectively) rather than the point at which ~50% of the developing embryos are seen extruding the first polar body. In addition, the holding temperature for fertilized eggs prior to treatment differed for both experiments (Scarpa *et al.* (1994) at 20°C and Beaumont and Kelly (1989) at 15 °C) and this will invariably alter the timing of second polar body extrusion. Exposing developing embryos to CB too early might allow some individuals to continue the extrusion of the second polar body post-exposure, while exposure to CB too late might miss some fast developing individuals which have already released the second polar body.

The most successful method of chemical 3N induction in *Mytilus sp.* besides CB exposure is by exposure to a considerably less toxic chemical 6-(Dimethylamino)purine (6-DMAP). Brake *et al.* (2002) found that the optimal method for induction (20°C, 400 µmol/l at 24 minutes post-fertilization) resulted in 83.1% 3N's. While 3N induction success and the resulting survival of M. *edulis* produced using this method is considerably less than what was found when using CB, further refinement of 6-DMAP induction techniques might result in a viable alternative to the highly toxic chemical, Cytochalasin B.

COMMERCIAL APPLICABILITY

There has been only sporadic interest in developing 3N *M. edulis* to fill gaps in the marketability of 2N mussels (Brake *et al.* 2004). According to Gordon King of Taylor

Resources Inc. (pers.comm.), they are not projecting an increase in the proportion of 6-DMAP induced 3N's beyond the 20% they already produce.

In short, successfully producing 3N *M. edulis* (100% induction), using a variety of induction methods (i.e., CB, 6-DMAP and heat shock, and possibly 4N x 2N crosses) in large quantities with comparable survival rates to 2N's appears to be quite viable. However, unlike some West coast *M. galloprovincialis* growers, the East coast *M. edulis* industry does not currently employ the large scale use of hatcheries to generate spat for grow-out. East coast growers currently rely heavily on wild spat collectors for generating seed. The high costs of running and sustaining a hatchery, the low wholesale price for *M. edulis* and the current success of spat collection are all prohibitive to the market production of 3N *M. edulis*. Despite current market conditions, there still appears to be a marketability niche in both Atlantic Canada and around the globe for 3N blue mussels to fill. While producing 3N's by CB induction is easily achieved, future regulations limiting CB's use by the aquaculture industry may make other methods of induction the only alternative.

REGULATORY CONSIDERATIONS

Currently, the introduction and transfer of 3N shellfish into either native or non-native habitat require that the shellfish in question undergo the same scrutiny required of 2N counterparts as imposed by the "National Code on Introductions and Transfers of Aquatic Organisms" (2002). The process begins when the applicant applies for the introduction of their 3N shellfish of interest through the Introductions and Transfers Committee Application Process. Firstly, the application is screened and additional information is provided by the applicant at the request of the committee. Secondly, the question is asked; "Is risk assessment necessary" for this organism based on historical genetic, ecological and disease management precedents? In addition, have there been recent "material changes" that might alter current assessment decisions? If historical precedents remain relevant, then the decision process begins and a written decision is provided to the applicant. If, based on lack of precedents, the Introductions and Transfers Committee believes that further investigation is needed to better assess the overall genetic, environmental, disease, cultural, economic, and social impacts, then a proper risk assessment will be conducted. Based on this broad reaching assessment and additional information from the applicant, a determination of overall risk (low, medium or high) and the procedures that might be employed to mitigate adverse outcomes will be supplied to the committee to assist in the decision making process.

According to Beaumont (2000) and Gaffney and Allen (1992), there are numerous pathological, ecological and genetic considerations to make with any introduction of foreign shellfish, be it 2N or 3N. First and foremost, hybridization studies should be conducted to determine the potential for, and the success of, hybridized animals. This involves determining whether there are any reproductively isolating mechanisms (RIM) (e.g. differences in spawning time, failure of hybrids to metamorphose, gametic incompatibility and geography) that would overcome potential hybridization with local species (Beaumont, 2000). Lack of RIM's, especially when utilizing aquaculture animals

that tend to have reduced genetic diversity, could lead to a loss in local species fitness. Fortunately, not only are 3N *M. galloprovincialis* gametogenically suppressed like other 3N induced species, they are also all male (Kiyomoto *et al.* 1996). Thus, assuming that these animals are sterile, this should act as a RIM that would negate the introgression of foreign genes and the production of hybrids.

Although there has been no official report of its occurrence with mussels, the progressive reversion from the 3N to the 2N state of ~20% as the animal matures is a potential problem in shellfish (Beaumont 2000; Nell 2002). If for some reason however, 3N's were to revert to 2N, there would be little likelihood of a population of *M. galloprovincialis* establishing itself in a non-native area when all induced 3N's are male to begin with. It is important to note that although reversion has been seen as a problem, nonetheless most reversions are not complete and the resulting heteroploid mosaic animals seldom produce haploid cells and are not likely viable (Allen *et al.* 1999). Even if the mussels were not to revert, under favourable environmental conditions it is possible for 3N *Mytilus (galloprovincialis)* to spawn, but sperm will not likely be viable and 3N's are only 2% as fecund as their diploid counterparts (Kiyomoto *et al.* 1996). As of yet no work has been done to explore reversion in *Mytilus edulis;* thus, ploidy confirmation of individual 3N mussels from our CB induction experiments should be conducted when subject animals are large enough to survive mantle tissue or hemolymph extraction for flow cytometric analysis. This slightly invasive procedure should be repeatedly performed with tissue from gametically mature individuals to record any potential instances of reversion over time.

No induction method, short of 4N x 2N crosses will produce 100% 3N individuals every time, and this leaves the potential of 2N individuals spawning and not only hybridizing with locally establish species, but establishing their own permanent community. In the case of triploid Pacific oysters, chemically induced 3N's are 2-3 times more likely to undergo reversion than their 3N counterparts produced by crossing 4N's by 2N's (Allen *et al.* 1999).

Furthermore, 3N's have been proposed as a mitigation tool to reduce the potential for foreign gene introgression into wild populations from genetically modified organisms (GMO's) containing novel gene constructs (Cogswell *et al.* 2002). As of yet in Canada, no GM fish or shellfish intended for aquacultural purposes have been approved for release beyond highly contained land based facilities (i.e., AquaBounty Fish Farms). The applications process is considerably more complex and must be in accordance with part 6 of the 1999 Canadian Environmental Protection Act. Considering the total lack of precedent and the current environmental, genetic, and social climate surrounding GMO's, any future application for the introduction of 3N transgenic shellfish through the Transfers and Introductions Committee will require extensive risk assessment that would likely result in a rejection of the application. While it is highly unlikely in the near future that with current 3N induction methods GM 3N shellfish will see open water, with the explosion of GM animals for medical research (Wright 2004) and food consumption, in conjunction with stringent containment procedures, 3N induction still remains the surest method to reproductively isolate organisms and further reduce the potential of foreign

gene introgression into wild populations. It is for this reason that researchers must continue to enhance current 3N induction techniques and develop efficient and cost effective novel means for restricting the reproductive capabilities in research or food organisms of interest.

Currently, through regulations laid out in the Food and Drugs Act, Health Canada is responsible for assessing the suitability of "novel" foods for human consumption. "Novel" as defined in Division 28 of the Food and Drugs Act and as applied to genetic and chromosomal manipulation is broadly defined as "a food that is derived from a plant, animal or microorganism that has been genetically modified such that: (i) the plant, animal or microorganism exhibits characteristics that were not previously observed in that plant, animal or microorganism, (ii) the plant, animal or microorganism no longer exhibits characteristics that were previously observed in that plant, animal or microorganism, or, (iii) one or more characteristics of the plant, animal or microorganism no longer fall within the anticipated range for that plant, animal or microorganism". While these definitions are quite broad and might or might not encompass chromosome set manipulation, some precedent utilizing triploid shellfish has been set (Nell, 2002; Gordon King, pers.comm. 2004). Nonetheless in Canada, when the intent is to market polyploidy shellfish for human consumption the manufacturer or importer must notify the Director General of the Food Directorate in writing of their intention to sell or advertise for sale the novel food. Only after an in-depth review of the pre-notification, "if the proposal is found acceptable by the committee, will the proponent be notified in writing by the Director General of the Food Directorate that the Department has no objection to the sale of the novel food in Canada". For more detailed information concerning the pre-notification process and recent revisions (July, 2003) to "Health Canada's Guidelines for the Safety Assessment of Novel Foods", visit http://www.hc-sc.gc.ca/food-aliment/mh-dm/ofb-bba/nfi-ani/e_consultation_main.html.

Finally, it will be important to continue the assessment (i.e., genetic, physiological, environmental, disease) of 3N shellfish to provide baseline data necessary for Introductions and Transfers Committees to make a proper evaluation of an organisms overall impact. In addition, 3N shellfish induction techniques and the associated aquacultural considerations (i.e., disease resistance, behaviour, physiology etc…) will be useful for aquaculturists when determining the best means for both producing and culturing 3N shellfish.

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APPENDICES

1. A histological section of a typical 3N male gonad.
2. Stocking density and feeding regime of 2N and 3N mussel buckets over the duration of growth parameter observations.

Appendix 1 – a micrograph of a typical stage II-b triploid *Mytilus edulis* gonad as described by Kiyomoto *et al.* 1996. Immature spermatogonia are present in most testicular acini as are phagocytizing haemocytes (green arrows). Also visible are the energy storing adipogranular cells (ADG cells) denoted by the blue arrows in the connective tissue.

Appendix 2 – Stocking density and feeding regime of 2N and 3N mussel buckets over the duration of growth parameter observations.

Date	Stocking Density (mussels/5L)	Algae Concentration cells/ml/day	Water Temperature (°C)
Jan 8th – Jan 26th	100	40,000 – 50,000	~16
Jan 26th – Feb 9th	100	80,000 – 180,000	~18
Feb 9th – Apr 23rd	44	180,000 – 600,000	~19
Apr 23rd – June 18th	25	600,000 – 1,200,000	~16.5
June 18th – July 20th	15	~1,200,000	~18.5

ACKNOWLEDGEMENTS

I would like to thank Amy Thompson, Shawn Roach, and Barry MacDonald for their assistance in maintaining the mussels over the duration of the experiments. A special thank you is also extended to Shawn Roach for his translation services and Ellen Kenchington, Lorraine Hamilton and Benedikte Vercaemer for their time spent reviewing this document. Also, I would like to personally thank Gordon King (Taylor Resources Inc., Washington State, U.S.A.) for his valuable input. This project was supported by the Canadian Biotechnology Strategy.